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Review

PEX Genes in Fungal Genomes: Common, Rare or Redundant

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PEX **genes encode proteins, termed peroxins, that are required for the biogenesis and proliferation of microbodies (peroxisomes). We have screened the available protein and DNA databases to identify putative peroxin orthologs in 17 fungal species (yeast and filamentous fungi) and in humans. This analysis demonstrated that most peroxins are present in all fungi under study. Only Pex16p is absent in most yeast species, with the exception of *Yarrowia lipolytica*, but this peroxin is present in all filamentous fungi. Furthermore, we found that the *Y. lipolytica PEX9* gene, a putative orphan gene, might encode a Pex26p ortholog. In addition, in the genomes of *Saccharomyces cerevisiae* and *Candida glabrata*, several *PEX* genes appear to have been duplicated, exemplified by the presence of paralogs of the peroxins Pex5p and Pex21p, which were absent in other organisms. In all organisms, we observed multiple paralogs of the peroxins involved in organelle proliferation. These proteins belong to two groups of peroxins that we propose to designate the Pex11p and Pex23p families. This redundancy may complicate future studies on peroxisome biogenesis and proliferation in fungal species.**

Key words: ***in silico*** **analysis, microbody, organelle biogenesis, peroxin, peroxisome**

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Microbodies (peroxisomes, glyoxysomes and glycosomes; in the remainder of this article designated peroxisomes) are involved in various important metabolic processes (1,2) and are essential in mammals, plants and *Trypanosomes* (3–5). So far, studies on peroxisomes have concentrated on a few yeast species, *Arabidopsis thaliana* and some mammalian systems (6). Most of the genes involved in peroxisome biogenesis, so-called *PEX* genes (7), were initially identified in studies with the yeast species *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris* and *Yarrowia lipolytica* (see Vizeacoumar et al. (8) and references therein). Mutants affected in these genes are usually characterized by the absence of normal peroxisomes in conjunction with mislocalization of peroxisomal matrix proteins to the cytosol. This results in an inability to perform specific biochemical reactions, e.g. the metabolism of fatty acids or methanol. In addition, certain *pex* mutants are exclusively affected in organelle proliferation.

Peroxisomes also play a vital role in filamentous fungi. In addition to fatty acid metabolism, the final steps of the biosynthesis of β-lactam antibiotics may take place in these organelles (9). Moreover, many filamentous fungi contain a special class of peroxisomes, the Woronin bodies, which are required to plug the septal pore on hyphal damage to prevent cytoplasmic leakage [(10,11); Figure 1]. Until now, in filamentous fungi, only a limited number of genes involved in peroxisome biogenesis and proliferation have been identified (12–17). Remarkably, mutants affected in these genes show phenotypes that have not been observed before in yeast species. Accordingly, *Podospora anserina pex2* mutants are affected in karyogamy, a process necessary for sexual sporulation (12), the *pex6* mutant of the plant pathogenic fungus *Colletotrichum lagenarium* is unable to infect plant cells (13) and the *Penicillium chrysogenum pex5* mutant is affected in asexual spore formation (16). These unexpected phenotypes of filamentous fungal *pex* mutants reflect the highly complex phenotypes of human peroxisome biogenesis disorders (PBDs) and $pex^{-/-}$ knockout mouse models (18). The current availability of genome sequences of various yeast species and filamentous fungi allowed identifying all currently known *PEX* genes in their genomes, enabling a direct comparison between these organisms with respect to peroxisome biogenesis and proliferation. Previously, the isolation of yeast *PEX* genes has been instrumental in the unraveling of the genetic defects in PBDs (19). Similarly, detailed studies on peroxisome biogenesis in filamentous fungi may become an important tool to understand the abnormal developmental processes that occur in certain human PBDs.

Analysis of Peroxins in Yeast Species and Filamentous Fungi

We investigated which genes encoding peroxins are present in the genomes of 15 fungal species (Table 1) by

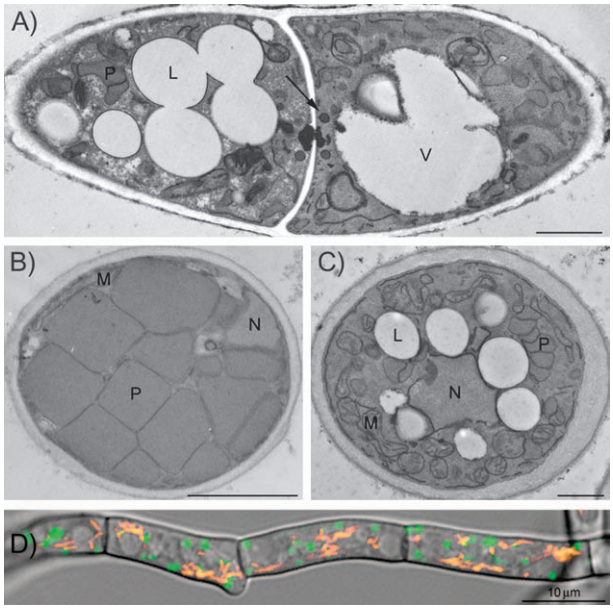

Figure 1: Induction of peroxisomes in yeast species and filamentous fungi. Peroxisomes can have highly variable sizes and shapes. Furthermore, they can be present in clusters but can also be dispersed throughout the cytoplasm. A) *Aspergillus tamarii* cell grown on oleate showing peroxisomes. In addition, Woronin bodies are present near the septum (arrow). Many lipid bodies are present. B) *Hansenula polymorpha* cell from a methanol-limited chemostat. More than 80% of the cell is filled with cuboid-shaped peroxisomes. C) *Saccharomyces cerevisiae* cell grown on oleate showing clustered peroxisomes. D) *Penicillium chrysogenum* hyphae producing the fluorescent peroxisomal protein green fluorescent protein-Ser-Lys-Leu-COOH (GFP-SKL). Cells were grown in penicillin-producing medium and treated with Mitotracker Orange to stain the mitochondria. The bar represents 1 μm, unless indicated otherwise. L, lipid body; M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole.

Table 1: Organisms analyzed in this study

	Abbreviation
Ascomycetes	
Saccharomycetaceae	
Saccharomyces cerevisiae S288C	Sc
Ashbya gossypii ATCC 10895 (*Eremothecium gossypii*)	Ag
Kluyveromyces lactis NRRL Y-1140	Kl
Debaryomyces hansenii CBS767	Dh
Pichia pastoris	Pp
Hansenula polymorpha (*Pichia angusta*)	Hp
Mitosporic Saccharomycetales	
Candida glabrata CBS138	Cg
Candida albicans SC5314	Ca
Dipodascaceae	
Yarrowia lipolytica CLIB122	Yl
Trichocomaceae	
Aspergillus fumigatus Af293	Af
Aspergillus nidulans FGSC A4	An
Penicillium chrysogenum Wis54-1255	Pc
Sordariomycetes	
Magnaporthe grisea 70-15	Mg
Neurospora crassa OR74A	Nc
Gibberella zeae PH-1 (*Fusarium graminearum*)	Gz
Schizosaccharomycete	
Schizosaccharomyces pombe 972h- (fission yeast)	Sp
Basidiomycetes	
Ustilaginomycete	
Ustilago maydis 521	Um
Hymenomycete	
Cryptococcus neoformans var. neoformans JEC21 (*Filobasidiella neoformans var. neoformans*)	Cn
Mammalia	
Homo sapiens (humans)	Hs

searching the databases of the National Center for Biotechnology Information (NCBI). We also included the *H. polymorpha* and *P. chrysogenum* genomes that will be made public soon [(20); DSM Anti-infectives, Delft, The Netherlands; unpublished data]. Furthermore, we have included single published peroxin sequences from fungi of which the genome sequence was not available at the NCBI. For direct comparison with higher eukaryotes, we also identified peroxins in the human genome. During these *in silico* analyses, we regularly observed incorrect translation products in the NCBI protein database (particularly in entries from filamentous fungi), presumably as a result of improper intron splicing. We cannot exclude the possibility that, because of this, in the basidiomycetes *Ustilago maydis* and *Cryptococcus neoformans* and in the fission yeast *Schizosaccharomyces pombe*, not all known peroxins could be identified. Only in specific cases, a comparison between sequences from closely related organisms allowed identification of the presumed correct intron positions (compare, e.g. accession numbers EAA61046 and EAL92562 with the sequences shown in Figure 3B). However, in many cases, the sequences are highly divergent (e.g. in many *U. maydis* and *C. neoformans* sequences), and only direct sequencing of complementary DNAs will provide the correct protein sequences. Also, in the human genome, we were not able to identify orthologs of all known peroxins. Many of these may not be present in human cells (see below). Additionally, certain peroxin sequences may have diverged too much to enable their identification as a functional homolog.

The results of our studies are summarized in Tables 2 and 3. Strikingly, the closely related yeast species *S. cerevisiae* and *Candida glabrata* contain a number of proteins with high similarity to certain peroxins (paralogs), which are absent in other organisms. This suggests that they may have resulted from gene duplications (21,22). Thus, both organisms contain a protein highly similar to Pex5p [*C. glabrata* Pex5Bp and *S. cerevisiae* Pex5Cp (Ymr018wp)] and paralogs of Pex21p (*S. cerevisiae* Pex18p and *C. glabrata* Pex21Bp) and Pex23p (*S. cerevisiae* Pex31p and *C. glabrata* Pex23Bp). Surprisingly, the mutual paralogs show remarkably little sequence similarity, which hints at

a diverged function. Consequently, we have included them as separate entries in Tables 2 and 3. In addition to this, the *S. cerevisiae* genome encodes a paralog of Pex25p (*S. cerevisiae* Pex27p), which is absent in all other fungi. In specific cases, it was demonstrated that in *S. cerevisiae*, the putative duplicated *PEX* genes show functional redundancy, as only deletion of both genes resulted in an unambiguous phenotype, e.g. Pex18p and Pex21p (23) and Pex23p, Pex31p and Pex32p (8). An exception to this is *S. cerevisiae* Pex5Cp (Ymr018wp), which does not seem to be involved in peroxisome biogenesis (24), while deletion of *Sc-PEX5* fully impairs import of a subset of peroxisomal matrix proteins (25). Also, the human genome encodes a protein highly similar to human PEX5, which was designated PEX5R [(24); Table 2]. Similar to PEX5, this protein contains tetratricopeptide repeat (TPR) domains, but it lacks the conserved N-terminal region (cf. Figure 2). PEX5R has been shown to interact with the peroxisomal-targeting signal (PTS) 1 that is present on most of the peroxisomal matrix proteins (see below). However, binding to other components of the translocation machinery was not observed, suggesting that this protein may actually have no role in peroxisomal matrix protein import. This is confirmed by the observation that mammalian *pex5* cell lines mislocalize all peroxisomal matrix proteins (26).

Peroxins Involved in the Formation of the Peroxisomal Membrane

So far, three *PEX* genes have been identified that are involved in the formation of the peroxisomal membrane (*PEX3*, *PEX16* and *PEX19*). In both yeast cells and human fibroblasts, deletion of any of these *PEX* genes causes the complete absence of remnant peroxisomal membrane structures [reviewed by Schliebs and Kunau (27)]. An exception to this rule is *Y. lipolytica pex19*, which contains structures resembling wild-type peroxisomes (28). This phenotype is not caused by functional redundancy because the *Y. lipolytica* genome only contains a single *PEX19* gene.

One of the main proposed functions of the interacting peroxins Pex19p and Pex3p is their role in sorting newly synthesized peroxisomal membrane proteins to their target membrane (27). As expected, both proteins are conserved from yeast to humans (Table 2). In addition, *Y. lipolytica* contains a paralog of Pex3p (Pex3Bp, with 28% identity to Yl-Pex3p). Deletion of *Yl-PEX3* resulted in a peroxisome-deficient phenotype (29), which implies that Yl-Pex3p and Yl-Pex3Bp are not fully functionally redundant. Nevertheless, a *Yl-pex3* null mutant still accumulated numerous small vesicles, a feature not normally observed in *pex3* mutants of other species, suggesting a possible role for Pex3Bp in their formation.

In mammalian cells, Pex16p is involved in the formation of the peroxisomal membrane (30). We show that Pex16p is present in all filamentous fungi but absent in most yeast species with the exception of *Y. lipolytica*. Remarkably, in this yeast, Pex16p appears to be involved in the regulation of peroxisome proliferation, rather than in the formation of (pre-) peroxisomes (31). The absence of Pex16p in other yeast species suggests that this function may be unique to *Y. lipolytica*. In addition to this, Brocard et al. (32) reported a role for microtubules and dynein motors in the formation of peroxisomal structures in human cells after reintroduction of *PEX16* in *pex16*-deficient fibroblasts. The observation that in most yeast species *PEX16* is absent may suggest that in these organisms, microtubules and dynein motors are not involved in the development of peroxisomes. So far, the role of Pex16p in filamentous fungi has not been investigated.

Matrix Protein Import into Peroxisomes; the PTS Receptors

Most peroxins are involved in the transport of matrix proteins from the cytosol into the peroxisome lumen. To this end, matrix proteins contain specific PTSs, designated PTS1 and PTS2, that are recognized by specific receptors [for review, see Purdue and Lazarow (6)]. The receptor Pex5p, that recognizes PTS1 proteins, is conserved from yeast to humans (Table 2). The C-terminus of Pex5p contains six TPR domains that are essential for binding the PTS1 [Figure 2B; (33)]. In addition, the genomes of the basidiomycetes *U. maydis* and *C. neoformans* also encode a Pex5p-related protein (designated Pex5/20p; for details, see below).

Initially, Pex7p was proposed to represent the sole PTS2 receptor. Pex7p is conserved from yeast to humans [but is absent in the nematode *Caenorhabditis elegans*; (34)]. However, in fungi, an accessory protein was identified that is required for recognition of PTS2 proteins. In *S. cerevisiae* and related yeast species, this is Pex21p, but in other yeast species and filamentous fungi, its functional homologue Pex20p (14,23,35–37). In the remainder of this article, we will exclusively use the term Pex20p for this accessory protein. Thus, in most fungi, the true PTS2 receptor is a complex between Pex7p and Pex20p. It is relevant to note here that Pex20p contains a Pex7p-binding site at its C-terminus. Additionally, the N-terminal region of Pex20p highly resembles the N-terminus of the PTS1 receptor Pex5p (Figure 2A,B). This structural similarity might explain why the basidiomycetes *U. maydis* and *C. neoformans* lack a separate Pex20p. In these organisms, a Pex5p/Pex20p fusion protein appears to be present. The N-terminus of this protein highly resembles the N-termini of both Pex20p and Pex5p (Figure 2A,B). Furthermore, a putative Pex7p-binding site is present, while the C-terminal region contains TPR domains similar to Pex5p (Figure 2B,C). This situation is clearly reminiscent of what is observed in higher eukaryotes. Mammalian cells contain two Pex5p proteins, designated PEX5S and PEX5L, resulting from alternative splicing. The larger form of the two proteins contains an extra loop that constitutes a Pex7p-binding site, which is

Table 2: Peroxins involved in the biogenesis of the peroxisomal membrane and in matrix protein import

	Ascomycetes											
	Yeasts									Other fungi		
	Sc[a]	Cg	Ag	Kl	Ca	Dh	Pp	Hp	Yl	Af	An	Pc
Pex1p	CAA82041	CAG60131	AAS53742	CAH02218	EAL02496	CAG89689	CAA85450	AAD52811	CAG82178	EAL93310	EAA57740	AAG09748
Pex2p	CAA89508	CAG60461	AAS50677	CAH00186	EAK95929	CAG85956	CAA65646	AAT97412	CAG77647	EAL88068	EAA58944	DQ793192
Pex3p	AAB64764	CAG62379	AAS52217	CAG99801	EAK94771	CAG89890	CAA96530	AAC49471	CAG78565	EAL91965	EAA64392	DQ793193
Pex3Bp	—	—	—	—	—	—	na	—	CAG83356	—	—	—
Pex4p	CAA97146	CAG60639	AAS53685	CAG99212	EAL03336	CAG87262	AAA53634	AAC16238	CAG79130	EAL87211	DNA1	DQ793194
Pex5p	CAA89730	CAG61665	AAS53824	CAH01742	EAK94251	CAG89098	AAB40613	AAC49040	CAG78803	EAL85289	EAA63772	AAR12222
Pex5Bp	—	CAG61076	—	—	—	—	na	—	—	—	—	—
Pex5Cp	CAA89120 (Ymr018wp)	—	—	—	—	—	na	—	—	—	—	—
Pex5/20p	—	—	—	—	—	—	na	—	—	—	—	—
Pex5Rp	—	—	—	—	—	—	na	—	—	—	—	—
Pex6p	AAA16574	CAG58438	AAS54884	CAG99125	EAK95956	CAG87108	CAA80278	AAD52812	CAG82306	EAL92776	EAA63496	AAG09749
Pex7p	CAA57183	CAG57936	AAS54301	CAG99215	EAK95226	CAG87150	AAC08303	ABA64462	CAG78389	EAL90870	EAA65909	DQ793195
Pex8p	CAA97079	CAG61238	AAS52889	CAH01253	EAK91777 + EAK91778[b]	CAG89446	AAC41653	CAA82928	CAG80447	EAL93137	EAA57947	DQ793196
Pex9p	ORF wrongly identified											
Pex10p	AAB64453	CAG62699	AAS53069	CAG99788	DNA3	CAG89101	AAB09086	CAA86101	CAG81606	EAL87045	EAA62774	DQ793197
Pex12p	CAA89129	CAG62649	AAS50837	CAG99378	EAL00707	CAG84342	AAC49402	AAM66157	CAG81532	EAL93972	EAA61357	DQ793198
Pex13p	AAB46885	CAG57840	AAS51456	CAG99931	EAK97421	CAG86337	AAB09087	DQ345349	CAG81789	EAL85282	EAA63824	DQ793199
Pex14p	AAS56829	CAG58828	AAS54871	CAG99440	EAK90926	CAG91028	AAG28574	AAB40596	CAG79323	EAL92562	EAA61046	DQ793200
Pex15p	CAA99046	CAG58938	AAS51506	CAG98135	—	—	na	—	—	—	—	—
Pex16p	—	—	—	—	—	—	na	—	CAG79622	EAL88469	EAA62294	DQ793201
Pex17p	CAA96116	CAG61398	AAS50595	CAH01010	EAK95385	CAG86168	AAF19606	DQ345350	CAG84025	See Pex14/17p		
Pex14/17p	—	—	—	—	—	—	na	—	—	EAL93590	EAA58642	DQ793202
Pex18p	AAB68992	—	—	—	—	—	na	—	—	—	—	—
Pex19p	CAA98630	CAG58359	AAS52741	CAG99258	EAK97275	CAG84799	AAD43507	AAK84070	AAK84827	EAL92487	EAA60977	DQ793203
Pex20p	—	—	—	—	EAK91603 + EAK94766[b]	CAG87898	AAX11696	AAX14715	CAG79226	EAL90176	EAA60479	DQ793204
Pex21p	CAA97267	CAG59241	AAS51769	CAG99735	—	—	na	—	—	—	—	—
Pex21Bp	—	CAG60281	—	—	—	—	na	—	—	—	—	—
Pex22p	AAC04978	CAG60970	AAS52329	CAG97800	EAK91040	CAG88727	AAD45664	DQ384616	CAG77876	—	—	—
Pex22p-like		—	—	—	—	—	na	—	—	EAL90994	EAA66006	DQ793205
Pex26p	—	—	—	—	EAK91093	CAG88929	na	DQ645588	DNA5	EAL93994	EAA61336	DQ793206

P, other published peroxin sequences; 1, *Podospora anserina* Car1p (Pex2p; CAA60739); 2, *Colletotrichum lagenarium* Pex6p (AAK16738); —, not present or not identifiable; na, full-genome sequence not available; DQ numbers indicate GenBank DNA accession numbers; DNA1, *Aspergillus nidulans* Pex4p sequence translated from GenBank accession number AACD01000130 [nucleotides (nt) 150195–150738, small ORF with intron]; DNA2, *Ustilago maydis* Pex4p sequence translated from accession number AACP01000006 (nt 97041–96550, small ORF with intron); DNA3, *Candida albicans* Pex10p sequence translated from accession number AACQ01000128 (nt 37281–36306, contains intron); DNA4, *Gibberella zeae* Pex22p-like sequence translated from accession number AACM01000080 (nt 4362–3039, one intron); DNA5, *Yarrowia lipolytica* Pex26p sequence translated from accession number NC_006072 (nt 117230–118387, represents the antisense sequence of the previously published *Y. lipolytica PEX9* gene).

[a]Abbreviations of the organisms are listed in Table 1.

[b]Partial ORFs encoded on nonoverlapping contigs.

absent in PEX5S [Figure 2D; (38)]. Plant cells only contain a single PEX5 that resembles mammalian PEX5L and has a PEX7-binding site (39). Thus, in mammals and plants, PTS1 and PTS2 import both require the action of PEX5, while in fungi, the presence of Pex20p may have allowed the PTS1 and PTS2 receptors to function more independently. Possibly, this has allowed fungi to adapt more quickly to changing environmental conditions.

Protein Translocation

Peroxisomal matrix proteins are usually imported into the peroxisomal lumen in a folded, oligomeric form. At the peroxisome, a large membrane-located complex is present that assists in docking and translocation of receptor-cargo moieties followed by recycling of the receptors (6). Most of the peroxins suggested to be involved in docking and translocation, namely the three RING finger proteins Pex2p, Pex10p and Pex12p and two proteins of the docking complex Pex13p and Pex14p, are conserved from yeast to humans (Table 2), stressing their importance in peroxisome biogenesis. Exceptions are Pex8p and Pex17p. Pex8p is the sole peroxin that resides in the peroxisome matrix. This peroxin not only is proposed to link the docking and RING finger complexes (40) but also functions in the release of PTS1 cargo proteins from their receptor (41). Pex8p is present in all fungi but has not been identified in higher eukaryotes.

Table 2: (*Extended*)

			Basidiomycetes				Mammalia
Other fungi					*Schizosaccharomyces pombe*		*Homo sapiens*
Mg	Nc	Gz	Um	Cn	Sp	P	Hs
XP_364454	EAA34641	EAA76787	EAK85195	AAW43248	CAA19256	—	EAL24149
XP_368589	EAA35361	EAA70670	EAK81310	AAW40683	CAA16981	1	AAH93043
XP_369909	EAA33751	EAA76989	EAK87104	AAW42444	CAB10141	—	AAH14551
—	—	—	—	—	—	—	—
XP_369064	EAA34737	EAA76379	DNA2	—	CAB91184	—	—
XP_360528	EAA36111	EAA68640	EAK83659	AAW46349	CAA22179	—	CAA59324 (PEX5L), CAA88131 (PEX5S)
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	EAK82973	AAW41849	—	—	—
—	—	—	—	—	—	—	AAH36183
XP_368715	EAA36040	EAA73732	EAK83459	AAW45333	CAB11501	2	AAH48331
XP_363555	AAN39560	EAA74171	EAK84499	AAW41119	P78798	—	AAH06268
XP_359449	EAA27783	EAA77627	EAK83936	AAW43468	CAB53406	—	—
XP_369099	EAA34967	EAA76761	EAK83811	AAW45079	CAB51769	—	AAH00543
XP_363845	EAA32773	EAA76413	EAK81282	AAW46724	CAD27496	—	AAH31085
XP_369087	EAA35785	EAA68396	EAK84395	AAW42381	CAB16740	—	AAH67090
XP_368216	EAA28304	EAA76904	EAK83123	AAW46857	CAA18656	—	AAH06327
—	—	—	—	—	—	—	—
XP_364166	EAA34648	EAA71849	EAK82801	AAW43797	CAA22819	—	AAH04356
See Pex14/17p				—	—	—	
XP_368163	EAA27748	EAA73655	EAK81127	—	—	—	—
—	—	—	—	—	—	—	—
XP_368273	EAA31855	EAA70162	EAK86072	AAW42876	CAA97344	—	AAH00496
XP_368606	AAN39561	EAA76911	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
XP_365689	EAA26537	DNA4	—	—	—	—	—
XP_359606	EAA28582	EAA76391	—	—	—	—	AAH47320

Pex17p, the third component of the proposed docking complex, has so far not been detected in higher eukaryotes, including humans. We observed that while all yeast genomes encode Pex17p, it seems to be lacking in filamentous fungi. However, further analyses identified a novel protein that we have designated Pex14/17p (Table 2). The N-terminus of this protein is similar to the highly conserved region present in the N-termini of Pex14ps (Figure 3), while the C-terminus of the protein shows weak similarity to that in yeast Pex17ps. Currently, the precise function of Pex17p in peroxisome biogenesis is unknown.

Peroxins implicated in recycling of the PTS receptors to the cytosol are Pex4p, a ubiquitin-conjugating enzyme (UBC), together with its membrane anchor Pex22p (42,43) and a complex containing the adenosine triphosphatases associated with various cellular activities (AAA ATPases) Pex1p and Pex6p with its membrane anchor Pex15p [in *S. cerevisiae*; (44)] or Pex26p [in mammals; (45)]. Remarkably, the proposed membrane anchors (Pex22p and Pex15p/Pex26p) appear to be much less conserved than the membrane-associated components Pex1p, Pex4p and Pex6p. Pex1p and Pex6p are conserved from yeast to humans. Orthologs of Pex4p, the sole UBC implicated in peroxisome biogenesis, can be identified in most fungi (with the exception of the basidiomycete *C. neoformans*; Table 2). Pex4p was also identified in *A. thaliana* (43) but not in mammalian cells. We initially identified the weakly conserved Pex4p-anchoring protein, Pex22p, only in yeast

Table 3: Peroxins involved in peroxisome proliferation

	Ascomycetes										
	Yeasts								Other fungi		
	Sc[a]	Cg	Ag	Kl	Ca	Dh	Hp	Yl	Af	An	Pc
A. Pex11p family											
Pex11p	CAA99168	CAG58440	AAS54890	CAG99119	EAK92906	CAG84534	DQ645582	CAG81724	EAL88627	EAA65086	AAQ08763
Pex11-2p	—	—	—	—	—	—	—	—	—	—	—
Pex11Bp	—	—	—	—	—	—	—	—	EAL88858	EAA62588	DQ793207
Pex11Cp	—	—	—	—	EAK96575	CAG85119	DQ645583	CAG81746	EAL94006	EAA61162	DQ793208
Pex11C2p	—	—	—	—	—	—	—	—	—	—	—
Pex11/25p	—	—	—	—	—	—	—	CAG81480	—	—	—
Pex25p	AAB68249	CAG60657	AAS54716	CAG97819	EAL00364	CAG85129	DQ645587	—	—	—	—
Pex27p	AAU09786	—	—	—	—	—	—	—	—	—	—
B. Pex23p family											
Pex23p	AAB64522 (=Pex30p)	CAG59269	AAS53871	CAH01863	EAL00808	CAG84593	DQ645584	CAG81562	EAL87054	EAA62785	DQ793209
Pex23Bp	—	CAG57703	—	—	—	—	—	—	—	—	—
Pex31p	CAA96987	—	—	—	—	—	—	—	—	—	—
Pex32p	CAA85129	CAG62586	AAS54705	CAH00149	EAK94878	CAG90534	DQ645590	—	—	—	—
Pex23p-like	AAS56780 (=Yer046wp)	—	AAS51815	CAG99225	EAL00164	CAG84886	DQ645585	CAG82241	EAL92588	EAA61073	DQ793210
Pex24p	AAB68980 (=Pex28p)	CAG60269	AAS51784	CAG99751	EAL04446	CAG85339	DQ645586	CAG80906	EAL87249	EAA61863	DQ793211
Pex29p	AAB64918	CAG62707	AAS52458	CAH02904	EAK96681	CAG88213	DQ645589	CAG78436	—	—	—

P, other published peroxin sequences; 1, *Candida boidinii* PMP30 (Pex11p; AAA85897); —, not present or not identifiable; DQ numbers indicate GenBank DNA accession numbers; DNA, *Magnaporthe grisea* Pex23p sequence; translation from accession number AACU02000486 (nucleotides 1–1823).
[a]Abbreviations of the organisms are listed in Table 1.

species. Similarly, use of the recently published *A. thaliana* Pex22p sequence (43) as query in PSI-BLAST screenings only revealed putative orthologs in plant species and other higher eukaryotes but not in mammals. In an effort to combine both groups of Pex22p orthologs, we repeated our analyses using exclusively the region of Pex22p believed to contain the Pex4p-binding site [corresponding to amino acids 64–158 of *Sc*-Pex22p; (43)]. PSI-BLAST analyses were performed on the eukaryote dataset of the nr protein database using an expect value of 1000. After each PSI-BLAST step, known Pex22p sequences were manually included in the next iteration. After exhaustive analysis with 16 identified Pex22p orthologs, we listed all fungal sequences among the first 250 hits. A comparison between the resulting hit lists revealed the presence of a set of highly similar proteins from filamentous fungi, which we have designated Pex22p-like proteins. This Pex22-like protein could not be identified in basidiomycetes and *S. pombe* (Table 2). Future investigations will elucidate whether this protein is the functional homologue of Pex22p.

Saccharomyces cerevisiae Pex15p and mammalian Pex26p are C-terminally anchored integral peroxisomal membrane proteins that bind Pex6p (44,45) and can therefore be considered functional homologs although they share almost no sequence identity (Figure 4). Analyses with baker's yeast Pex15p only identified orthologs in highly related yeast species (Table 2). PSI-BLAST searches for orthologs of human Pex26p resulted in the identification of Pex26p in various filamentous fungi. Subsequent Genomic BLAST analyses using these putative fungal Pex26ps as queries identified a DNA sequence designated *PEX9* in the *Y. lipolytica* genome (46), but the similarity to Pex26p was exclusively observed with a translation product from the opposite DNA strand of *PEX9*. This putative *Y. lipolytica* translation product showed similarity to putative *Candida albicans*, *Debaryomyces hansenii* and *H. polymorpha* Pex26 proteins. Thus, all eukaryotes seem to contain an ortholog of either Pex15p or Pex26p (Table 2; Figure 4). We were unable to identify a protein similar to Pex26p in basidiomycetes and *S. pombe*, which is presumably due to the very weak similarity between these proteins or by possible incorrect intron splicing.

Our analyses suggested that the *Yl-PEX9* gene encodes a Pex26p ortholog. Until now, Yl-Pex9p seemed to represent an orphan peroxin that had no orthologs in other species (46). Possibly, the proposed open reading frame (ORF) may not represent the correct coding sequence of the *PEX9* gene. Comparison of the published DNA sequence with that of the *Y. lipolytica* CLIB122 genome revealed a frame shift in the *PEX9* coding sequence. To strengthen our analyses, we have resequenced the *PEX9* region from two *Y. lipolytica* strains [E122 and PO1d; (46,47)]. The sequences obtained were completely identical to that of the *Y. lipolytica* CLIB122 genome. It must be noted that the strategy used to delete the *Yl-PEX9* ORF (46) also deletes the proposed *PEX26* ORF. The observation that this deletion resulted in a phenotype typical for

Table 3: *(Extended)*

			Basidiomycetes				Mammalia
Other fungi					*Schizosaccharomyces pombe*		*Homo sapiens*
Mg	Nc	Gz	Um	Cn	Sp	P	Hs
AAX07686	EAA31192	EAA76922	EAK85343	AAW40784	CAB46672	1	AAH09697 (Pex11α),
–	–	EAA70837	–	–	–	–	AAH11963 (Pex11β),
XP_368596	EAA28424	EAA76892	–	–	–	–	AAH08780 (Pex11γ)
XP_359506	EAA33643	EAA67776	EAK81351	AAW44574	CAA93808	–	
–	–	EAA71255	–	–	–	–	
–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–
DNA	EAA31290	EAA72143	EAK82619	AAW40681	–	–	–
–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–
–	–	–	–	–	CAA19113	–	–
XP_367286	EAA29611	EAA70083	EAK82006	AAW40897	CAB36510	–	–
XP_368485	EAA31634	EAA68322	EAK84137	AAW43076	CAB44773	–	–
–	–	–	–	–	–	–	–

peroxisome-deficient mutants confirms that this gene indeed encodes a *bona fide* peroxin.

Proliferation of Peroxisomes

Peroxins implicated in peroxisome proliferation are listed in Table 3. In all cases, these proteins are integral protein components of the peroxisomal membrane. We have classified these peroxins in two families based on their (weak) similarity to either Pex11p or Pex23p.

Pex11p family

Orthologs of Pex11p are present in all eukaryotes. So far, overproduction of Pex11p has induced massive proliferation of peroxisomes in all organisms studied, including humans. Conversely, deletion of *PEX11* in baker's yeast significantly reduced organelle numbers (17,48–50). The genome of *S. cerevisiae* encodes a single Pex11 protein, while filamentous fungi contain three Pex11p isoforms, designated Pex11p, Pex11Bp and the less similar Pex11Cp (Table 3). The yeast species *C. albicans*, *D. hansenii*, *H. polymorpha* and *Y. lipolytica* also contain the previously unidentified Pex11Cp, which is absent in *S cerevisiae* and its close relatives. Remarkably, the wheat pathogen *Gibberella zeae* appears to contain not only Pex11p, Pex11Bp and Pex11Cp but also paralogs of Pex11p and Pex11Cp (Table 3). The situation in filamentous fungi resembles that observed in higher eukaryotes, which also contain multiple forms of Pex11p. Human cells contain three Pex11p-related proteins [PEX11α, β and γ; Table 3; for review, see Thoms and Erdmann (51)]. Of these, PEX11α appears to be responsible for peroxisome proliferation in response to external stimuli, while PEX11β is required for constitutive peroxisome proliferation. Pex11p from filamentous fungi shows highest similarity to both mammalian isoforms α and β, while Pex11Bp is much less similar. Analogous to Pex11Cp in fungi, mammalian PEX11γ shows least similarity to the other two mammalian isoforms. A role of fungal Pex11Cp and mammalian PEX11γ in peroxisome proliferation remains to be established.

In addition to Pex11p, most yeast species contain a protein with very low similarity to Pex11p that was designated Pex25p (52,53). A clear-cut Pex25p homologue could not be identified in *Y. lipolytica*. However, this organism contains a protein with weak similarity to both Pex11p and Pex25p that may be functionally related to Pex25p (Table 3). Filamentous fungi and human cells also seem to lack a Pex25p homologue, but these organisms contain multiple Pex11p isoforms that may perform a similar function. In *S. cerevisiae*, Pex25p was shown to interact with its partially redundant paralog, designated Pex27p, that is absent in other species (Table 3). Similar to Pex11p overproduction, overexpression of *PEX25* or *PEX27* in *S. cerevisiae* resulted in peroxisome proliferation. Conversely, mutants deleted for either of these genes contained fewer, enlarged organelles. *S. cerevisiae* Pex25p recruits the guanosine triphosphatase Rho1p to the

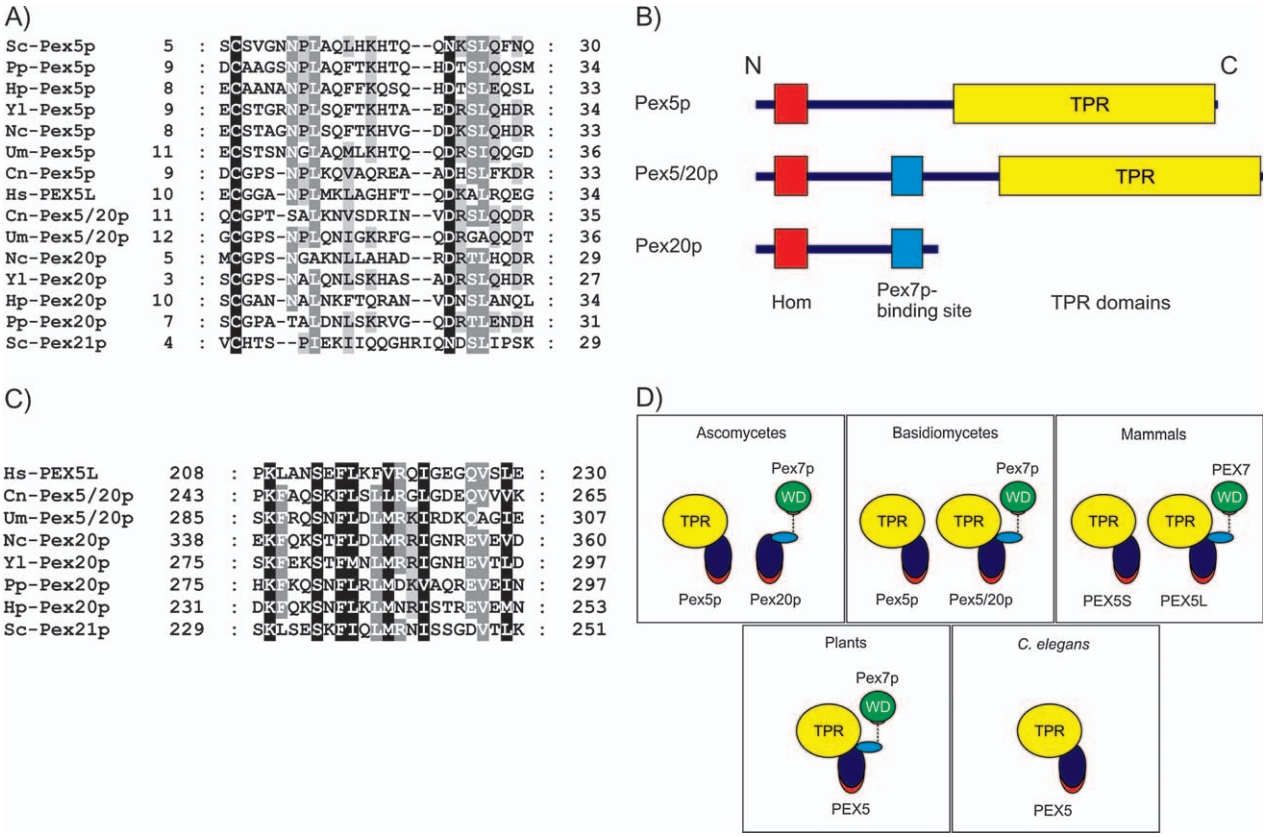

Figure 2: Analysis of Pex5p, Pex5/20p and Pex20p. A) Sequence alignment of the conserved N-terminal regions of human PEX5L and selected fungal Pex5ps, Pex5/20ps and Pex20ps. The abbreviations of organisms are given in Table 1. The sequence accession numbers are given in Table 2. Sequences were aligned using the CLUSTAL_X program (65). Gaps were introduced to maximize the similarity. Residues that are similar in all proteins are shaded black. Similar residues in at least twelve of the proteins are shaded dark gray, while those that are similar in at least nine of the proteins are shaded light gray. B) Schematic representation of Pex5p, Pex5/20p and Pex20p. The homologous N-terminal domains (Hom) are indicated in red. The putative Pex7p-binding domains are indicated in blue, while the TPR domains are indicated in yellow. C) Sequence alignment of the putative Pex7p-binding domain in human PEX5L, basidiomycete Pex5/20ps, selected fungal Pex20ps and *Saccharomyces cerevisiae* Pex21p. The abbreviations of organisms are given in Table 1. The sequence accession numbers are given in Table 2. Sequences were aligned using the CLUSTAL_X program. Residues that are similar in all proteins are shaded black. Similar residues in at least six of the proteins are shaded dark gray, while those that are similar in at least five of the proteins are shaded light gray. D) Schematic representation of PTS receptors in different organisms. Ascomycetes contain both Pex5p and Pex20p enabling the PTS1 and PTS2 routes to be independent from each other. Basidiomycetes contain two genes that encode Pex5p and Pex5/20p, the latter of which contains a Pex7p-binding site. In mammalian cells, a single *PEX5* gene produces two forms of PEX5S (PEX5 and PEX5L) by differential splicing. PEX5L is required for both PTS1 and PTS2 import, making the PTS2 import route fully dependent on the *PEX5* gene. In plants, a single PEX5 protein is present that contains a PEX7-binding site. Also, here PTS2 import is fully dependent on *PEX5*. In the nematode *Caenorhabditis elegans*, the PTS2 pathway is absent and peroxisomal proteins only have a PTS1. The small homologous N-terminal domains of Pex5p, Pex5/20p and Pex20p are indicated in red. The putative Pex7p-binding domain is indicated in blue, while the TPR domains are indicated in yellow. The WD40 repeats of the PTS2 receptor Pex7p are indicated in green.

peroxisomal membrane (54). It has been suggested that Rho1p binding might regulate actin assembly on the peroxisomal membrane, thereby controlling organelle membrane dynamics and biogenesis.

Pex23 family

A second family of proteins involved in peroxisome proliferation consists of two groups, the members of which are weakly similar. Based on similarity, the first group consists of the peroxin Pex23p and related proteins. These proteins contain a DysF motif with an unknown function that was first observed in human dysferlin [SMART motifs SM00693 and SM00694; (55)]. The second group is characterized by Pex24p and the related peroxin Pex29p, which also show weak similarity to the DysF domain. So far, these peroxins have been the subject of only few studies (8,56–58). BLAST analyses suggest that *S. cerevisiae* Pex30p is the ortholog of *Y. lipolytica* Pex23p, while *S. cerevisiae* Pex28p is presumably the ortholog of *Y. lipolytica* Pex24p. For practical purposes, we will only use the *Y. lipolytica* nomenclature for these two peroxins.

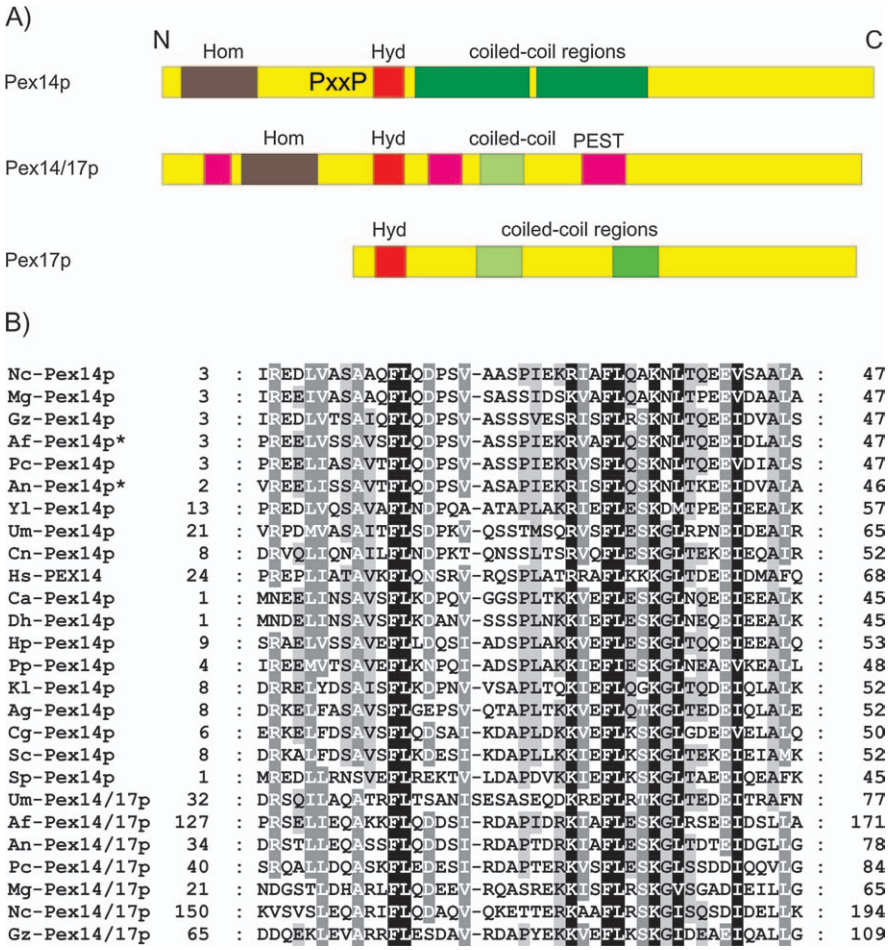

Figure 3: Analysis of fungal Pex14p and Pex14/17p. A) Schematic representation of fungal Pex14ps and Pex14/17ps. The homologous N-terminal domains (Hom) are indicated in brown. A hydrophobic region possibly representing a membrane attachment site is indicated in red. Putative coiled-coil regions are indicated in green (dark green, very significant; light green, weakly significant). Putative PEST sequences, indicating possible recognition sites for protein turnover, are indicated in purple. PxxP represents a ligand for the SH3-domain of Pex13p, the binding partner of Pex14p on the peroxisomal membrane. B) Sequence alignment of the conserved N-terminal regions of human PEX14, fungal Pex14ps and Pex14/17ps. The abbreviations of organisms are given in Table 1. The sequence accession numbers are given in Table 2. Sequences were aligned using the CLUSTAL_X program. Gaps were introduced to maximize the similarity. Residues that are similar in all proteins are shaded black. Similar residues in at least 21 of the proteins are shaded dark gray, while those that are similar in at least 15 of the proteins are shaded light gray. The asterisks indicate the *Aspergillus fumigatus* and *Aspergillus nidulans* Pex14p sequences that required correction based on sequence comparisons with Pex14ps from other fungi.

Our analyses show that in all fungi, orthologs of Pex23p are present (with *S. pombe* being a possible exception). *Cg*-Pex23Bp and *Sc*-Pex31p are presumably redundant paralogs of Pex23p that are absent in other yeast species and filamentous fungi. A second member of the Pex23p group is Pex32p. Orthologs of this peroxin are only present in yeast (including *S. pombe*) but not in filamentous fungi. The role of Pex23p, Pex31p and Pex32p in peroxisome homeostasis is not yet clear. *Y. lipolytica pex23* mutants cannot grow on oleate and partially mislocalize peroxisomal proteins (56). In contrast to this, *S. cerevisiae* Pex23p, Pex31p and Pex32p are exclusively required for peroxisome proliferation. *Sc*-Pex23p appears to be a positive regulator of peroxisome size, while *Sc*-Pex31p and *Sc*-Pex32p negatively regulate this process. Human cells do not appear to have Pex23p, Pex31p or Pex32p orthologs. Nevertheless, a number of proteins with a DysF motif can be identified in the human genome. Some of these may play a hitherto unknown role in peroxisome proliferation.

In addition to the published members of this group of the Pex23p family, we identified one other yeast protein related to Pex23p with a DysF domain that is conserved in yeast species (but not in *C. glabrata*) and in filamentous fungi (Table 3). The size of this protein is rather variable. In *S. cerevisiae*, *C. albicans* and *D. hansenii*, this protein is rather small (amino acids 143–172), relative to its counterpart in other yeast and filamentous fungi. An *S. cerevisiae* mutant lacking this protein (*yer046w/spo73*; (59)) is affected in spore wall formation during sporulation, which

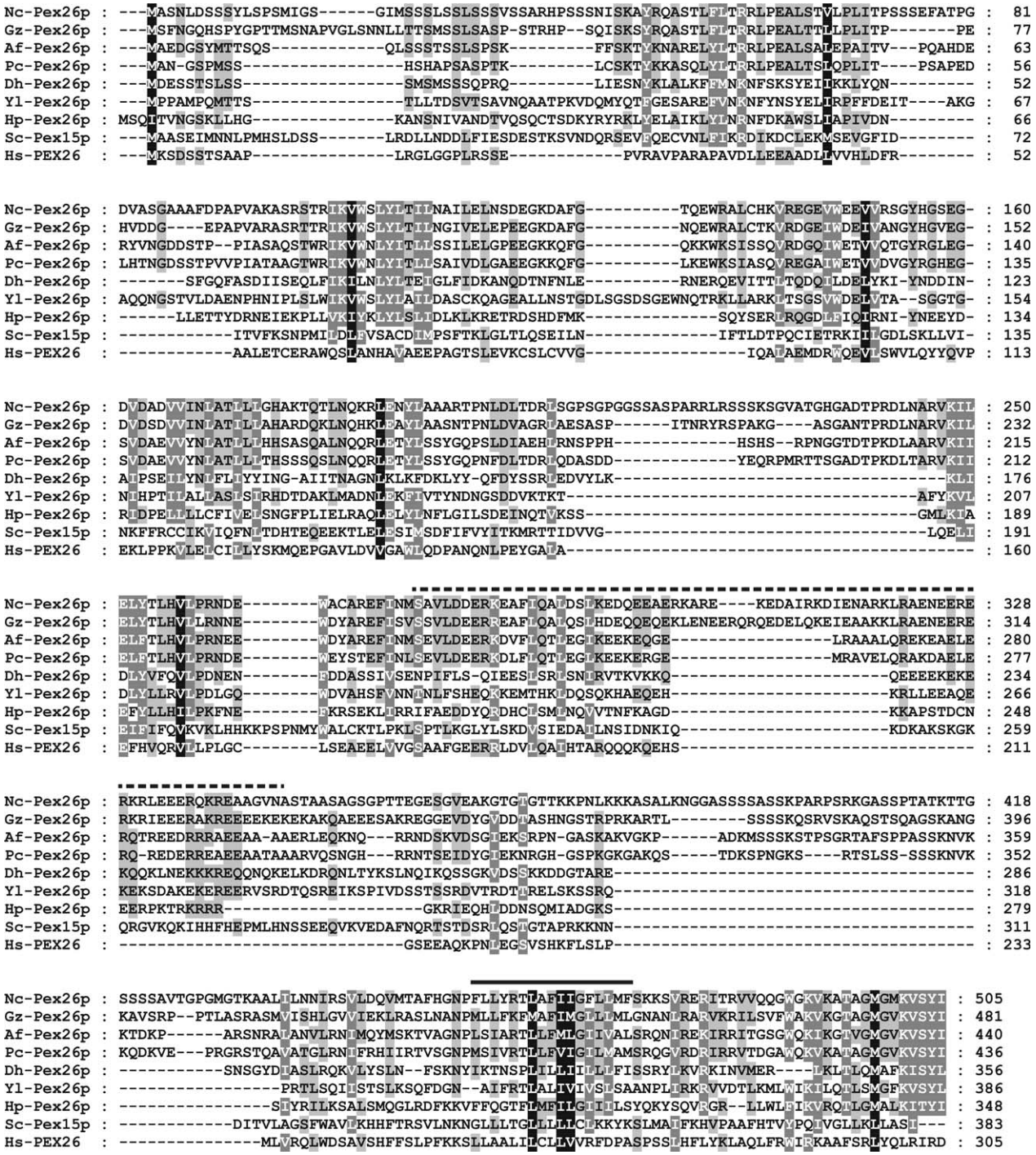

Figure 4: Sequence alignment of *Saccharomyces cerevisiae* Pex15p, human PEX26 and selected yeast/fungal Pex26ps. The abbreviations of organisms are given in Table 1. The sequence accession numbers are given in Table 2. The Gz-Pex26p sequence was taken from the *Gibberella zeae* genome sequence (GenBank AACM01000273.1, nucleotides 40 597–42 090 with one intron removed) based on sequence comparison with other Pex26p sequences from filamentous fungi. Sequences were aligned using the CLUSTAL_X program. Gaps were introduced to maximize the similarity. Residues that are similar in all proteins are shaded black. Similar residues in at least seven of the proteins are shaded dark gray, while those that are similar in at least five of the proteins are shaded light gray. The solid line above the sequences indicates the C-terminal membrane anchor. The dashed line above the sequences indicates a putative coiled-coil region that was observed in most Pex26ps but not in human PEX26, *Hansenula polymorpha* Pex26p and *S. cerevisiae* Pex15p. The significance of this sequence is unknown.

suggests that it may not be important in peroxisome proliferation.

The second group of peroxins in the Pex23p family consists of Pex24p and Pex29p. These peroxins are conserved in all yeast species. Remarkably, the genomes of filamentous fungi (and the fission yeast *S. pombe*) encode only a single protein with similarity to both Pex24p and Pex29p. For clarity, we have designated these proteins Pex24p. Also, the role of Pex24p and Pex29p in peroxisome homeostasis is not yet clear. Similar to *pex23* mutants, *Y. lipolytica* and *S. cerevisiae* mutants deleted in *PEX24* show significantly different phenotypes. In *S. cerevisiae*, cells deleted for either *PEX24* or *PEX29* (or both) have a phenotype consistent with a role for these *PEX* genes in controlling peroxisome separation and multiplication (58). In contrast, *Y. lipolytica pex24* mutants show a defect in peroxisomal protein translocation (57). Orthologs of Pex24p (and Pex29p) have not been identified in higher eukaryotes, including humans.

Concluding Remarks

Our data indicate that almost all peroxins identified so far are conserved in yeast and filamentous fungi. In specific cases, genome duplication has resulted in the presence of two or more related proteins (paralogs). This is especially the case for peroxins involved in peroxisome proliferation as exemplified by the presence of multiple members of the Pex11p and Pex23p families. The presence of such paralogs is obvious in all evaluated fungal species and even in human cells. However, in the yeast species *S. cerevisiae* and *C. glabrata* (and in an exceptional case also *Y. lipolytica*), gene duplication has probably also resulted in the presence of a number of paralogs of peroxins involved in peroxisome formation. Because such duplications may possibly result in functional redundancy (e.g. Pex18p/Pex21p; (23)), this complicates the use of these organisms in the identification of novel genes involved in peroxisome biogenesis.

Our data also indicate that the peroxin numbering may no longer be accurate because some numbers actually represent similar peroxins in different species. Related to this is that the proposed *Y. lipolytica PEX9* ORF probably does not represent the correct reading frame. It may be considered to rename *PEX9* (into *PEX26*). In addition, renumbering of *PEX28* (into *PEX24*) and *PEX30* (into *PEX23*) would fit these genes better in the unified nomenclature.

Our BLAST analyses suggest that the total number of peroxins that may be considered to be the minimal requirement for peroxisome biogenesis/matrix protein import in fungi amounts to 17 (Pex1–8, 10, 12–14, 17, 19, 20, 22 and 26p). However, other hitherto unidentified proteins, which are either redundant or essential, may be involved in the organelle formation as well. Based on its function in mammalian cells, Pex16p may also play an – hitherto unknown – essential role in peroxisome biogenesis in filamentous fungi. However, this peroxin is absent in most yeast species and its role in *Y. lipolytica* seems related to organelle proliferation. Actually, Pex16p is one of only few peroxins that is not conserved from yeast to humans. Others are Pex4p and Pex22p that were not detected in mammals but were identified in *A. thaliana* (43) and Pex8p and Pex17p that seem to be absent in all higher eukaryotes, including humans.

Despite the strong conservation of the peroxisome biogenesis machinery, the sequence similarity among peroxins is in some cases extremely low (e.g. Pex8p, Pex17p and Pex22p). Therefore, we anticipate that orthologs for some peroxins may still be uncovered in humans. It is, however, possible that specific peroxins may not be conserved at all. This possibility is based on the observation that the distribution and movement of peroxisomes are highly variable and mediated by either microtubuli or actin filaments, depending on the species under study. In mammalian cells, peroxisomes move via dynein/kinesin motors along microtubuli [reviewed by Schrader et al. (60)]. Recently, it was demonstrated that microtubules and dynein motors are also required in the formation of preperoxisomes in mammalian cells (32). In yeast species (and certain plants), peroxisomes move via the Myo2p motor along actin microfilaments, and this Myo2p/actin-based movement is required for organelle inheritance (61–63), a process that also appears to require the peroxin Pex19p (64). So far, studies on peroxisome movement in filamentous fungi are lacking. Analogous to the proposed role for microtubules and dynein motors in peroxisome formation in human cells, the (actin) cytoskeleton in yeast (and possibly also in filamentous fungi) may significantly contribute to peroxisome biogenesis. Such a scenario would imply that the proteins that connect peroxisomes to the cytoskeleton may in fact be peroxins. Because binding of peroxisomes to the cytoskeleton is species dependent, these proteins are not expected to be conserved in all species. This is clearly uncharted territory that needs further investigation.

Finally, our data suggest that in certain aspects, peroxisome biogenesis in filamentous fungi may better reflect mammalian cells than yeast cells. This is exemplified by the identification of Pex16p and multiple forms of Pex11p in all filamentous fungi and by the presence of a PEX5L-related protein (Pex5/20p) in basidiomycetes. This supports the notion that a better understanding of peroxisome biogenesis in filamentous fungi may be helpful to explain the highly complex phenotypes of PBDs in humans.

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